

INHIBITOR PROTEINS OF A PROTEASE AND USE THEREOF

FIELD OF THE INVENTION

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The present invention relates to a chimeric inhibitor protein of a protease comprising an inhibiting polypeptidic sequence and at least one polypeptidic sequence of a substrate-enzyme interaction site specific for a protease.

10 Other objects of the invention are to provide a purified and isolated DNA sequence encoding the chimeric inhibitor protein of a protease, an expression vector characterized in that it comprises said purified and isolated DNA sequence, a eukaryotic or prokaryotic host cell transformed with this expression vector and a method of producing a chimeric inhibitor protein.

BACKGROUND OF THE INVENTION

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Of all proteins expressed by living organisms, proteases are among the most critical in mediating pathways of cell life and death. In fact, the initial interactions between protease and substrate and subsequent cleavage lie at the base of a vast spectrum of essential biological events including thrombosis, coagulation and apoptosis.

20 Dysregulated proteolysis, or imbalance between proteases and antiproteases, has been searched intensively based on the suspicion that it could be a key factor in many pathologies where proteases have been involved such as cancer, autoimmune diseases, inflammation and infectious diseases. Different studies done with antiproteolytic agents in cancer and inflammatory disease (such as rheumatoid arthritis and emphysema) models have also shown
25 interesting outcome improvement, strengthening the antiproteolytic therapy and the role of balance between proteases and antiproteases.

For example, in prostate cancer, which is one of the most common diagnosed cancers in American men, proteases are believed to play a pivotal role in the malignant behaviour of cancer cells including rapid tumor growth, invasion, and metastasis.

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Human glandular kallikrein (hK2) protein is a trypsin-like serine protease expressed predominantly in the prostate epithelium. Firstly isolated from human seminal plasma, hK2 has recently emerged as a diagnostic marker for prostate cancer (*Deperthes et al. 1995*

“Isolation of prostatic kallikrein hK2, also known as hGK-1, in human seminal plasma”
Biochim Biophys Acta 1245, 311-6).

Beside its role as marker, its proteolytic activities suggest that hK2 could contribute to cancer progression. Several potential functions for this enzyme have been proposed, including the activation of urokinase-type plasminogen activator and inactivation of plasminogen activator inhibitor-1, activation of pro-PSA, degradation of fibronectin and degradation of insulin-like growth factor binding protein (IGF-BP) (for review see *Cloutier et al.*, 2004 “Development of recombinant inhibitors specific to human kallikrein 2 using phase-display selected substrates” *Eur J Biochem* 3, 607-13).

It has recently been shown that kallikrein hK2 can form a specific complex with a protease inhibitor, known as PI-6, in cancers and particularly in prostate cancer. Based on the discovery of this specific complex, US Patents 6,284,873 and 6,472,143 provide a diagnostic method for determining the presence or absence of cancer or tissue necrosis.

Taking into account its prostate tissue specific expression and the involvement of all its potential substrates in cancer development, hK2 is also considered as a potential therapeutic target (*Darson et al.* 1997 “Human glandular kallikrein 2 (hK2) expression in prostatic intraepithelial neoplasia and adenocarcinoma: a novel prostate cancer marker” *Urology* 49, 857-62). Therefore, the development of specific and long-lasting protease inhibitors and especially kallikrein inhibitors would be useful.

These Protease inhibitor candidates can be selected among the serpin (serine protease inhibitors) family, which is a large family of proteins implicated in the regulation of complex physiological processes. These proteins of about 45 kDa can be subdivided into two groups, one being inhibitory and the other non-inhibitory.

Serpins contain an exposed flexible reactive-site loop or reactive-serpin loop (RSL), which is implicated in the interaction with the putative target proteinase. Following the binding to the enzyme and cleavage of the P1-P1’ scissile bond of the RSL, a covalent complex is formed (*Huntington et al.* 2000 “Structure of a serpin-protease complex shows inhibition by deformation” *Nature* 407, 923-6). Formation of this complex induces a major conformational rearrangement and thereby traps irreversibly the target protease. The inhibitory specificity of serpins is largely attributed to the nature of the residues at P1-P’1 positions and the length of the RSL. Changing the RSL domain or the reactive site of serpins is one

approach to understand the inhibitory process between a serpin and an enzyme and to develop specific inhibitors (*Dufour et al.* 2001 "The contribution of arginine residues within the P6-P1 region of alpha 1-antitrypsin to its reaction with furin" *J Biol Chem* 276, 38971-9 and *Plotnick et al.* 2002 "The effects of reactive site location on the inhibitory properties of the serpin alpha(1)-antichymotrypsin" *J Biol Chem* 277, 29927-35).

Several serpins such as protein C inhibitor, $\alpha 2$ antiplasmin, antithrombin-III, $\alpha 1$ -antichymotrypsin (ACT), or protease inhibitor 6 have been identified as hK2 inhibitors (*Saedi et al.* 2001 "Human kallikrein 2 (hK2), but not prostate-specific antigen (PSA), rapidly complexes with protease inhibitor 6 (PI-6) released from prostate carcinoma cells" *Int J Cancer* 94, 558-63). The relatively slow complex formation between hK2 and ACT is mainly attributed to residues Leu 358-Ser 359 at P1-P'1 positions of the RSL, an unfavourable peptide bond for this trypsin-like enzyme.

Up to now, only selections of new kallikrein inhibitors, which specifically inhibit plasma kallikrein, and use thereof in therapeutic and diagnostic methods have been disclosed (patents US 6,057,287, US 6,333,402, US 5,994,125, and US 5,795,865). However, these patents describe the production of inhibitors that are homologous to bovine pancreatic trypsin inhibitor Kunitz domains, and especially proteins that are homologous to lipoprotein-associated coagulation inhibitor (LACI) Kunitz domains, which specifically inhibit plasma kallikreins.

Besides being specific for plasma kallikrein, these inhibitors are quite small molecules and bind to plasma kallikrein in a reversible manner. One of the major drawback of this approach is that the use of proteins inhibiting their targets in a reversible manner bears the risk that decomplexation of the protease restores its activity.

Therefore, one advantage of using larger inhibitors, as described herein, is that this leads to the formation of covalent complexes which inhibits the protease target in an irreversible manner. A further advantage of the present invention is that large covalent complexes are known to be quickly eliminated from circulation.

SUMMARY OF THE INVENTION

Therefore, the object of the present invention is to provide a protease inhibitor protein
5 with high specificity for said protease and use thereof in a pharmaceutical composition. This
inhibitor protein is chimeric insofar as it comprises an inhibiting polypeptidic sequence and at
least one polypeptidic sequence of a substrate-enzyme interaction site specific for a protease.

An other object of the invention is to provide a purified and isolated DNA sequence
10 encoding the chimeric inhibitor protein of a protease, an expression vector characterized in
that it comprises said purified and isolated DNA sequence and a eukaryotic or prokaryotic
host cell transformed with this expression vector.

A further object of the present invention is to provide a method for producing the
15 chimeric inhibitor protein of a protease. This method comprises the steps of

- a) selecting a polynucleotidic sequence encoding a substrate-enzyme interaction site
specific for a protease,
- b) introducing said polynucleotidic sequence into a sequence encoding an inhibitor
protein of a serine or cysteine protease, so as to obtain a chimeric sequence,
- 20 c) allowing expression of said chimeric sequence in a cell expression system under
suitable conditions,
- d) and recovering the chimeric inhibitor protein of a protease.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 represents an SDS-PAGE analysis under reducing conditions of purified recombinant ACT. Variant 6.1 (lane 1) and wild type ACT (lane 2).

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Figure 2 shows the stoichiometry of inhibition (SI) of hK2 by rACT_{WT} and its variants. The SI was determined using linear regression analysis to extrapolate the I/E ratio (i.e. the x intercept).

10 **Figures 3 A and B** show the formation of complex between hK2 and recombinant inhibitors. Arrows indicate hK2 (E), inhibitor (I), and hK2-ACT complex (E-I).

Figures 4 A and B show the inhibition of hK2 by rACT_{WT} and its variants under pseudo-first order conditions. The interaction of hK2 and recombinant serpins was measured under
15 pseudo-first order conditions using progress curve method.

Figure 5 corresponds to the determination of the purity of the inhibitors by SDS-PAGE analysis under reducing conditions of inhibitors developed in examples 1 and 2.

20 **Figures 6 A and B** show a Western Blot analysis of the inhibitory reaction between recombinant ACT and the human kallikreins hK2 (Figure 6A) and PSA (Figure 6B).

Figure 7A shows the DNA and protein sequences of MD820

25 **Figure 7B** shows the DNA and protein sequences of MD62

Figure 7C shows the DNA and protein sequences of MD83

Figure 7D shows the DNA and protein sequences of MD67

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Figure 7E shows the DNA and protein sequences of MD61

Figure 7F shows the DNA and protein sequences of MD518

35 **Figure 7G** shows the DNA and protein sequences of MDCI

Figure 8 represents a comparison of RSL sequences of ACT and MD inhibitors. Plain type residues are common to ACT_{WT}, bold and underlined residues correspond to mutation in RSL of ACT variants. The putative cleavage site in serpins is marked by asterisks between the P1-P1' residues.

Figure 9 represents the pQE9 expression vector map.

Figure 10A shows the Inhibition of tumor growth by MD 62. Prostate cancer cells DU-145 (3 x 10⁶ cells), transfected with human kallikrein 2, were implanted in nude mice and then treated with MD 62 (5 or 25 µg/injection).

Figure 10B shows the inhibition of tumor growth by MD 67. Prostate cancer cells DU-145 (3 x 10⁶ cells), transfected with human kallikrein 2, were implanted in nude mice and then treated with MD 67 (5 or 25 µg/injection).

Figure 11 shows the inhibition of tumor growth by MD CI. Prostate cancer cells DU-145 (3 x 10⁶ cells), transfected with human kallikrein 2, were implanted in nude mice and then treated with MD CI (5 or 50 µg/injection).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a chimeric inhibitor protein of a protease comprising an inhibiting polypeptidic sequence and at least one polypeptidic sequence of a substrate-enzyme interaction site specific for said protease.

“Chimeric inhibitor protein” refers to a protein comprising two or more polypeptides, which are from different origins, i.e. which do not occur together in the nature.

As used herein, the terms “protein”, “polypeptide”, “polypeptidic”, “peptide” and “peptidic” are used interchangeably herein to designate a series of amino acid residues connected to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues.

The chimeric protein of the invention is an inhibitor of a protease and is composed of an inhibiting polypeptidic sequence and of, at least, one polypeptidic sequence of a substrate-enzyme interaction site specific for said protease. The polypeptidic sequence of a substrate-enzyme interaction site confers highly selective properties of the inhibitor towards a particular protease and this polypeptidic sequence is selected on the basis of the protease to be inhibited.

Typically, this polypeptidic sequence of a substrate-enzyme interaction site can be a substrate active site sequence. "Substrate active site sequence" refers to a sequence found on a substrate and which is a preferential recognition site for a protease. Recognition of the substrate active site sequence by a protease can lead to the activation, inactivation or degradation of the substrate and most of the time this high affinity interaction involves the recognition not only of a specific sequence but also of its 3-D conformation.

Encompassed by the present invention is also a molecular chimera of the substrate active site sequence. By "molecular chimera" is intended a polynucleotide sequence that may include a functional portion of the substrate active site sequence and that will be obtained, for example, by protein chemistry techniques known by those skilled in the art.

Particular combinations of the substrate active site sequence or fragments or subportions thereof are also considered in the present invention.

"Fragments" refer to sequences sharing at least 40% amino acids in length with the respective sequence of the substrate active site. These sequences can be used as long as they exhibit the same properties as the native sequence from which they derive. Preferably these sequences share more than 70%, preferably more than 80%, in particular more than 90% amino acids in length with the respective sequence the substrate active site.

These fragments can be prepared by a variety of methods and techniques known in the art such as for example chemical synthesis.

The present invention also includes variants of the substrate active site sequence. The term "variants" refer to polypeptides having amino acid sequences that differ to some extent from a native sequence polypeptide, that is amino acid sequences that vary from the native sequence by conservative amino acid substitutions, whereby one or more amino acids are substituted by another with same characteristics and conformational roles. The amino acid sequence variants possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of the native amino acid sequence. Conservative amino acid substitutions are herein defined as exchanges within one of the following five groups:

- I. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro, Gly
- II. Polar, positively charged residues: His, Arg, Lys
- III. Polar, negatively charged residues: and their amides: Asp, Asn, Glu, Gln
- IV. Large, aromatic residues: Phe, Tyr, Trp
- 5 V. Large, aliphatic, nonpolar residues: Met, Leu, Ile, Val, Cys.

10 Preferably the substrate of the present invention is a serpin, in this case the substrate active site sequence may be a Reactive Serpin Loop sequence, fragments thereof, a molecular chimera thereof, a combination thereof and/or variants thereof.

“Reactive Serpin Loop” or “Reactive Site Loop” or RSL refers to an exposed flexible reactive-site loop found in serpin and which is implicated in the interaction with the putative target protease. From the residue on the amino acid side of the scissile bond, and moving away from the bond, residues are conventionally called P1, P2, P3, *etc.* Residues that follow the
15 scissile bond are called P1', P2', P3', *etc.* Usually, the RSL is composed of 6 to 12 amino acid residues.

This RSL sequence can be selected from the group comprising the SEQ ID No 16, 17, 18, 19, 20, 21 and 22, fragments thereof, molecular chimeras thereof, combinations thereof and/or variants thereof.

20 RSL sequence may also be selected among the following possibilities shown in table I.

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Table I
Positions in the RSL which can be changed

| POSITION IN THE REACTIVE SITE LOOP | | | | | | | | |
|------------------------------------|----|----|----|----|-----|-----|-----|-----|
| P5 | P4 | P3 | P2 | P1 | P'1 | P'2 | P'3 | P'4 |
| | | S | S | R | T | E | | |
| | | K | T | R | S | N | | |
| | I | S | P | R | S | | | |
| | G | V | F | K | S | | | |
| | D | T | V | K | S | | | |
| | B | T | K | R | S | | | |
| | | L | G | R | S | L | | |
| | | R | G | R | S | E | | |
| | | | R | R | S | I | D | |
| | | V | L | R | S | P | | |
| | | P | F | R | S | G | | |
| | | | | R | S | | S | V |
| | A | N | A | R | S | | | |
| | | S | D | R | T | A | | |
| | | K | L | R | T | T | | |
| | | | | R | A | A | M | M |
| | | | T | R | A | P | M | |
| | | D | V | R | A | A | | |
| | | P | G | R | A | P | | |
| | V | E | S | R | A | | | |
| | | | A | R | A | S | E | |
| | Y | L | Q | R | V | | | |
| | R | L | E | R | V | | | |
| | | | E | R | V | S | P | |
| | S | S | P | R | V | | P | Y |
| | | | | R | V | G | | |
| | P | S | A | R | M | | | |
| | | E | G | R | M | A | | |
| | | T | V | K | M | P | | |
| | | | L | R | M | F | T | |
| | | | H | R | M | S | E | |
| | | | | R | P | Q | E | L |
| | | | V | R | P | L | | |
| | | S | G | R | L | A | | |
| | D | T | L | R | F | | | |
| | | Q | W | R | N | S | | |
| | | | | R | N | D | K | L |
| | | | M | R | N | L | S | |
| | | | T | R | D | S | R | |
| | | G | S | R | D | | | |
| | | M | S | R | Q | | | |
| | | Q | H | R | Q | M | G | |
| | | T | S | R | | | | |
| | | Q | S | R | | | | |
| | | T | S | K | | | | |
| | | F | R | K | I | | | |

Amino acid sequence of P4-P3' residues in RSL (Reactive Serpin Loop) corresponding to potential substrate peptide

Blank spaces indicate that there is no modification needed in order to obtain substrate specificity to hK2.

Usually the protease is selected from the group comprising kallikrein, chymotrypsin (Chtr), urokinase (uPA) and human neutrophil elastase (HNE) enzymes. Preferably, the protease is a human kallikrein, most preferably this human kallikrein is hK2 (also known as hGK-1).

HK2 belongs to the kallikrein gene family which is composed of 15 members but only the Prostate Specific Antigen (PSA or hK3) and hK2 are expressed at a high level by the

prostate. One of the potential physiologic role of hK2 is the proteolytic degradation of the sperm-entrapping gel formed immediately after ejaculation, particularly the cleavage of semenogelins and fibronectin. In addition, it was demonstrated in an *in vitro* test that hK2 can enhance insulin-like growth factor's (IGF) mitogenic action by IGF binding protein hydrolysis. *In vitro* studies also showed that hK2 activates prourokinase, generate bradykinin-like substances from kininogens (potential cross-activation of EGF-receptors via B2 bradykinin receptors) and converts proPSA into an active form. These hK2 activities represent arguments in favor of a potential role of hK2 in extracellular matrix protein degradation and consequently detachment and migration of prostate cancer cells. In addition, hK2 could enhance prostate cancer development by release of mitogenic factor and activation of growth receptors.

In case the protease to be inhibited is a cystein protease, then this protease is selected from the group comprising cathepsins (K, L, and S subtypes), the prohormone thiol proteinase and the caspase family (Caspases 1, 3, 4, and 8).

Cystein proteases, which are proteolytic enzymes that utilize a cystein residue for their catalytic activity, can be grouped in at least 30 protein families. Each family contains proteins with similar amino acid sequences and evolutionarily conserved sequence motifs which reflects the family member's similar 3D structures.

The inhibiting polypeptidic sequence of the chimeric inhibitor protein is usually a serine or a cysteine protease.

In the case the inhibiting polypeptidic sequence is from a serine protease then this inhibiting polypeptidic sequence is preferably a serpin sequence, fragments thereof, a molecular chimera thereof, a combination thereof and/or variants thereof.

This serpin sequence can be selected from the group comprising the α -1 antichymotrypsin (ACT), protein C inhibitor (PCI), α -1 antiproteinase (AAT), human α -1 antitrypsin-related protein precursor (ATR), α -2-plasmin inhibitor (AAP), human anti-thrombin-III precursor (ATIII), protease inhibitor 10 (PI10), human collagen-binding protein 2 precursor (CBP2), protease inhibitor 7 (PI7), protease inhibitor leuserpin 2 (HLS2), human plasma protease C1 inhibitor (C1 INH), monocyte/neutrophil elastase inhibitor (M/NEI), plasminogen activator inhibitor-3 (PAI3), protease inhibitor 4 (PI4), protease inhibitor 5 (PI5),

protease inhibitor 12 (PI12), human plasminogen activator inhibitor-1 precursor endothelial (PAI-1), human plasminogen activator inhibitor-2 placental (PAI2), human pigment epithelium-derived factor precursor (PEDF), protease inhibitor 6 (PI6), protease inhibitor 8 (PI8), protease inhibitor 9 (PI9), human squamous cell carcinoma antigen 1 (SCCA-1), human squamous cell carcinoma antigen 2 (SCCA-2), T4-binding globulin (TBG), Megsin, and protease inhibitor 14 (PI14), fragments thereof, molecular chimeras thereof, combinations thereof and/or variants thereof.

Since most of these serpins have different names, we include below a table summarizing their specifications:

Table II

| Serpin | Accession Number | RSL sequence |
|--|------------------|-----------------------|
| PI or AAT, A1AT_HUMAN ALPHA-1-ANTITRYPSIN PRECURSOR (ALPHA-1 PROTEASE INHIBITOR) (ALPHA-1-ANTIPROTEINASE) | sp P01009 | GTEAAGAMFLEAIPMSIPPE |
| PIL or ATR, A1AU_HUMAN ALPHA-1-ANTITRYPSIN-RELATED PROTEIN PRECURSOR | sp P20848 | GTEATGAPHLEEKAWSKYQT |
| PLI OR AAP, A2AP_HUMAN ALPHA-2-ANTIPLASMIN PRECURSOR (ALPHA-2-PLASMIN INHIBITOR) (ALPHA-2-PI) (ALPHA-2-AP) | sp P08697 | GVEAAAATSIAMSRMSLSFF |
| AACT, AACT_HUMAN ALPHA-1-ANTICHYMOTRYPSIN PRECURSOR (ACT) | sp P01011 | GTEASAATAVKITLLSALVE |
| AT3, ANT3_HUMAN ANTITHROMBIN-III PRECURSOR (ATIII) | sp P01008 | GSEAAASTAVVIAGRSLNPN |
| PI10, BOMA_HUMAN BOMAPIN (PROTEASE INHIBITOR 10) | sp P48595 | GTEAAAGSGSEIDIRIRVPS |
| CBP2, CBP2_HUMAN COLLAGEN-BINDING PROTEIN 2 PRECURSOR (COLLIGIN 2) | sp P50454 | GNPFDQDIYGREELRSPKLF |
| PI7 or PN1, GDN_HUMAN GLIA DERIVED NEXIN PRECURSOR (GDN) (PROTEASE NEXIN I) (PN-1) (PROTEASE INHIBITOR 7) | sp P07093 | GTKASAATTAILIARSSPPW |
| HCF2, HEP2_HUMAN HEPARIN COFACTOR II PRECURSOR (HC-II) (PROTEASE INHIBITOR LEUSERPIN 2) (HLS2) | sp P05546 | GTQATTVTTVGFMPLSTQVR |
| C1NH or C1IN, IC1_HUMAN PLASMA PROTEASE C1 INHIBITOR PRECURSOR (C1 INH) | sp P05155 | GVEAAAASAI SVARTLLVFE |
| ELANH2 or PI2, ILEU_HUMAN LEUKOCYTE ELASTASE INHIBITOR (LEI) (MONOCYTE/NEUTROPHIL ELASTASE INHIBITOR) (M/NEI) (EI) | sp P30740 | GTEAAAATAGIATFCMLMPE |
| PCI or PLANH3 or PROCI, IPSP_HUMAN PLASMA SERINE PROTEASE INHIBITOR PRECURSOR (PCI) (PROTEIN C INHIBITOR) (PLASMINOGEN ACTIVATOR INHIBITOR-3) (PAI3) | sp P05154 | GTRAAAATGTIFTFRSARLN |
| PI4 or KST, KAIN_HUMAN KALLISTATIN PRECURSOR (KALLIKREIN INHIBITOR) (PROTEASE INHIBITOR 4) | sp P29622 | GTEAAAATTFKIFSAQTN |
| PI5, MASP_HUMAN MASPIN PRECURSOR (PROTEASE INHIBITOR 5) | sp P36952 | GGDSIEVPGARILQHKDELN |
| PI12, NEUS_HUMAN NEUROSERPIN PRECURSOR (PROTEASE INHIBITOR 12) | sp Q99574 | GSEAAVSGMIAISRMVLY |
| PAI1 or PLANH1, sp P05121 PAI1_HUMAN PLASMINOGEN ACTIVATOR INHIBITOR-1 PRECURSOR, ENDOTHELIAL (PAI-1) | sp P05121 | GTVASSSTAVIVSARMAPEE |
| PAI2 or PLANH2, PAI2_HUMAN PLASMINOGEN ACTIVATOR INHIBITOR-2, PLACENTAL (PAI-2) (MONOCYTE ARG- SERPIN) | sp P05120 | GTEAAAGTGGVMTGRTGHGG |

| | | |
|---|-----------------------------|----------------------|
| (UROKINASE INHIBITOR) | | |
| PEDF, PEDF_HUMAN PIGMENT EPITHELIUM-DERIVED FACTOR PRECURSOR (PEDF) (EPC-1) | sp P36955 | GAGTTPSPGLQPAHLTFPLD |
| PI6 or PTI, PTI6_HUMAN PLACENTAL THROMBIN INHIBITOR (CYTOPLASMIC ANTIPROTEINASE) (CAP) (PROTEASE INHIBITOR 6) | sp P35237 | GTEAAAATAAIMMRCARFV |
| PI8, PTI8_HUMAN CYTOPLASMIC ANTIPROTEINASE 2 (CAP2) (CAP-2) (PROTEASE INHIBITOR 8) | sp P50452 | GTEAAAATAVVRNSRCSRME |
| PI9, PTI9_HUMAN CYTOPLASMIC ANTIPROTEINASE 3 (CAP3) (CAP-3) (PROTEASE INHIBITOR 9) | sp P50453 | GTEAAAASSCFVVAECMES |
| SCCA1, SCC1_HUMAN SQUAMOUS CELL CARCINOMA ANTIGEN 1 (SCCA-1) (PROTEIN T4-A) | sp P29508 | GAEAAAATAVVGFGSSPAST |
| SCCA2, SCC2_HUMAN SQUAMOUS CELL CARCINOMA ANTIGEN 2 (SCCA-2) (LEUPIN) | sp P48594 | GVEAAAATAVVVVELSSPST |
| TBG, THBG_HUMAN THYROXINE-BINDING GLOBULIN PRECURSOR (T4-BINDING GLOBULIN) | sp P05543 | GTEAAAVPEVELSDQPENTF |
| MEGSIN | gi 4505149 ref N P_003775.1 | GTEATAATGSNIVEKQLPQS |
| PI14, pancpin, TSA2004 | gi 3724282 db B AA33766.1 | GSEAATSTGIHIPVIMSLAQ |

As an example of chimeric inhibitor proteins according to the invention, Applicants have surprisingly found 6 new chimeric inhibitor proteins specific for the protease hK2 as resumed below in table III, these inhibitors are:

TABLE III

| Chimeric inhibitors | Other name | SEQ ID N° (protein) |
|----------------------|------------|---------------------|
| rACT _{8,20} | MD820 | 2 |
| rACT _{6,2} | MD62 | 4 |
| rACT _{8,3} | MD83 | 6 |
| rACT _{6,7} | MD67 | 8 |
| rACT _{6,1} | MD61 | 10 |
| ACT _{5,18} | MD518 | 12 |

These chimeric inhibitor proteins have been obtained by modifying the RSL of α 1-antichymotrypsin (rACT), which is known to inhibit a large panel of human enzymes such as chymotrypsin, mast cell chymase, cathepsin G, prostatic kallikreins hK2 and PSA (hK3), in order to change the specificity of this serpin. Peptide sequences, selected as substrates for the enzyme hK2 by phage display technology as explained in detail in Example 1, have been used to replace the scissile bond and neighbour amino acid residues of the RSL. Recombinant inhibitors were produced in bacteria and purified by affinity chromatography.

Compared to wild type rACT, which inhibited hK2 very slowly (12-16h), the modified rACTs have been shown to form a covalent complex very quickly within few minutes. Three of the six rACT variants were specific to hK2 with high association constants (see tables V and VI). Incubating with an excess of inhibitors ($[I]_0/[E]_0$ of 100:1) for 30 minutes, hK2 is completely inhibited by rACT_{6,2}, rACT_{8,3}, rACT_{6,7} and rACT_{6,1}, whereas rACT_{8,20} and rACT_{5,18} inhibited 95 % and 73 % of enzyme activity, respectively. Under this condition, wild type rACT showed no inhibition activity toward hK2. Among these variants, two (rACT_{8,3} and rACT_{5,18}) are specific to hK2, inhibiting no other tested enzyme. Two other variants, rACT_{6,7} and rACT_{6,2}, inhibited as well PK at 36 % and 100% respectively. As wild-type ACT, variant rACT_{8,20} inhibited the two chymotrypsin-like proteases Chtr and PSA but additionally also PK and HNE. None of the recombinant serpins showed inhibitory activity against the kallikrein hK1 and uPA.

Additionally, applicants have also found that replacing residues P3-P3' located in RSL structure of rACT_{WT} by substrate pentapeptide coding for the RSL of Protein C inhibitor (PCI) lead to the production of a chimeric inhibitor (MDCI) which is able to inhibit kallikreins hK2 and hK3.

Therefore, the chimeric inhibitor of a protease may be selected from the group comprising MD820, MD62, MD61, MD67 and MDCI. Preferably this chimeric inhibitor protein is MD62 or MD61.

It is known that a Stoichiometry of Inhibition (SI) value superior to one is generally interpreted as substrate behaviour of serpin. In this scheme, after formation of an initial Michaelis complex and cleavage in the RSL, most of the complex is broken down into active enzyme and cleaved inhibitor which is definitively inactivated. Applicants have analyzed ACT variants-hK2 reactions for non complex forming cleavage of the inhibitor, incubating the samples at a 10:1 fold excess of inhibitor to protease. These conditions, close to or below calculated SI values of the tested ACT variants (see Table VI), normally favour proteolysis of serpins or serpin-protease complexes. Surprisingly, Applicants have observed a discrepancy to this hypothesis since degradation of variant ACTs by hK2 was not observed despite high SI values. Without wishing to be bound by the theory, a possible explanation for the lack of ACT degradation is the condition under which the SI determination was performed. Covalent ACTs-hK2 complexes are forming *in vitro* very slowly. This is in agreement with our

observation that after 30 minutes incubation at 25°C no inhibition of hK2 with wild-type ACT has been detected (Table V) and that even after prolonged incubation at 37°C, hK2 was only partially complexed with wild type ACT (Figure 3).

Applicants have also assessed the specificity of these new inhibitors toward other proteases. The evaluation has been performed under the same conditions for all proteases (pseudo-physiological conditions) in order to ensure a better translation for further *in vivo* applications. The permutation of RSL cleavage site for hK2 phage display selected substrates has changed wild type ACT into highly sensitive inhibitors for hK2. In addition, two of these inhibitors showed a unique reactivity with hK2 and not with other studied enzymes known to target similar biological substrates, such as plasma kallikrein, hK1, PSA, urokinase (uPA), and human neutrophil elastase (HNE). To Applicants knowledge, this is the first report mentioning the development of a specific inhibitor for hK2.

Interestingly, rACT_{8.20} (MD820) inhibits beside hK2 also chymotrypsin, and more weakly plasma kallikrein and human elastase, representing a broad inhibition specificity.

The inhibiting polypeptidic sequence of the chimeric inhibitor protein may also be selected from a cysteine protease since there are now a number of well-documented instances of inhibition of cysteine proteases by serpins (*Gettins P.G.W., 2002 "Serpins structure, mechanism, and function" in Chem. Rev, 102, 4751-4803*). These examples include inhibition of cathepsins K, L and S by the serpin squamous cell carcinoma antigen1, inhibition of prohormone thiol proteinase by the α -1 antichymotrypsin, and inhibition of members of the caspase family, including caspase 1 (interleukine 1 β converting enzyme), caspase 3, and caspase 8 by the viral serpin crmA and caspases 1, 4 and 8 by the human serpin PI9.

When recombinant techniques are employed to prepare a chimeric inhibitor protein of a protease in accordance with the present invention, nucleic acid molecules or fragments thereof encoding the polypeptides are preferably used.

Therefore the present invention also relates to a purified and isolated DNA sequence encoding the chimeric inhibitor protein of a protease as described above.

"A purified and isolated DNA sequence" refers to the state in which the nucleic acid molecule encoding the chimeric inhibitor protein of a protease of the invention, or nucleic acid

encoding such chimeric inhibitor protein of a protease will be, in accordance with the present invention. Nucleic acid will be free or substantially free of material with which it is naturally associated such as other polypeptides or nucleic acids with which it is found in its natural environment, or the environment in which it is prepared (e. g. cell culture) when such
5 preparation is by recombinant DNA technology practised *in vitro* or *in vivo*.

DNA which can be used herein is any polydeoxynucleotide sequence, including, e.g. double-stranded DNA, single-stranded DNA, double-stranded DNA wherein one or both strands are composed of two or more fragments, double-stranded DNA wherein one or both
10 strands have an uninterrupted phosphodiester backbone, DNA containing one or more single-stranded portion(s) and one or more double-stranded portion(s), double-stranded DNA wherein the DNA strands are fully complementary, double-stranded DNA wherein the DNA strands are only partially complementary, circular DNA, covalently- closed DNA, linear DNA, covalently cross-linked DNA, cDNA, chemically- synthesized DNA, semi-synthetic DNA,
15 biosynthetic DNA, naturally-isolated DNA, enzyme-digested DNA, sheared DNA, labeled DNA, such as radiolabeled DNA and fluorochrome-labeled DNA, DNA containing one or more non-naturally occurring species of nucleic acid.

DNA sequences that encode the chimeric inhibitor protein of a protease, or a fragment thereof, can be synthesized by standard chemical techniques, for example, the phosphotriester
20 method or via automated synthesis methods and PCR methods.

The purified and isolated DNA sequence encoding the chimeric inhibitor protein according to the invention may also be produced by enzymatic techniques. Thus, restriction enzymes, which cleave nucleic acid molecules at predefined recognition sequences can be used to isolate nucleic acid sequences from larger nucleic acid molecules containing the
25 nucleic acid sequence, such as DNA (or RNA) that codes for the chimeric inhibitor protein or for a fragment thereof.

Encompassed by the present invention is also a nucleic acid in the form of a polyribonucleotide (RNA), including, e.g., single-stranded RNA, double- stranded RNA,
30 double-stranded RNA wherein one or both strands are composed of two or more fragments, double-stranded RNA wherein one or both strands have an uninterrupted phosphodiester backbone, RNA containing one or more single-stranded portion(s) and one or more double-

stranded portion(s), double-stranded RNA wherein the RNA strands are fully complementary, double-stranded RNA wherein the RNA strands are only partially complementary, covalently crosslinked RNA, enzyme-digested RNA, sheared RNA, mRNA, chemically-synthesized RNA, semi-synthetic RNA, biosynthetic RNA, naturally-isolated RNA, labeled RNA, such as radiolabeled RNA and fluorochrome-labeled RNA, RNA containing one or more non-naturally- occurring species of nucleic acid.

The purified and isolated DNA sequence encoding a chimeric inhibitor of a protease is preferably selected from the group comprising SEQ ID N° 1, SEQ ID N° 3, SEQ ID N° 5, SEQ ID N° 7, SEQ ID N° 9, SEQ ID N° 11 and SEQ ID N° 13.

The present invention also includes variants of the aforementioned sequences, that is nucleotide sequences that vary from the reference sequence by conservative nucleotide substitutions, whereby one or more nucleotides are substituted by another with same characteristics.

Yet another concern of the present invention is to provide an expression vector comprising the purified and isolated sequence encoding the chimeric inhibitor protein of a protease as described above. The choice of an expression vector depends directly, as it is well known in the art, on the functional properties desired, e.g., chimeric inhibitor protein expression and the host cell to be transformed or transfected.

Additionally, the expression vector may further comprise a promoter operably linked to the purified and isolated DNA sequence. This means that the linked isolated and purified DNA sequence encoding the chimeric inhibitor protein of a protease of the present invention is under control of a suitable regulatory sequence which allows expression, i.e. transcription and translation of the inserted isolated and purified DNA sequence.

As used herein, the term "promoter" designates any additional regulatory sequences as known in the art e.g. a promoter and/or an enhancer, polyadenylation sites and splice junctions usually employed for the expression of the polypeptide or may include additionally one or more separate targeting sequences and may optionally encode a selectable marker. Promoters which can be used provided that such promoters are compatible with the host cell are e.g. promoters obtained from the genomes of viruses such as polyoma virus, adenovirus (such as Adenovirus 2), papilloma virus (such as bovine papilloma virus), avian sarcoma virus,

cytomegalovirus (such as murine or human cytomegalovirus immediate early promoter), a retrovirus, hepatitis-B virus, and Simian Virus 40 (such as SV 40 early and late promoters) or promoters obtained from heterologous mammalian promoters, such as the actin promoter or an immunoglobulin promoter or heat shock promoters.

5 Enhancers which can be used are e.g. enhancer sequences known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin) or enhancer from a eukaryotic cell virus. e.g. the SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma, and adenovirus enhancers.

10 A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e. g., E. coli plasmids col E1, pCRI, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAs, e. g., the
15 numerous derivatives of phage X, e. g., NM989, and other phage DNA, e. g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

20 Most preferably the expression vector is pQE-9.

Another concern of the present invention is to provide a eukaryotic or prokaryotic host cell transformed or transfected with an expression vector described herein.

25 The term "cell transfected" or "cell transformed" or "transfected/transformed cell" means the cell into which the extracellular DNA has been introduced and thus harbours the extracellular DNA. The DNA might be introduced into the cell so that the nucleic acid is replicable either as a chromosomal integrant or as an extra chromosomal element.

30 Transformation or transfection of appropriate eukaryotic or prokaryotic host cells with an expression vector comprising a purified an isolated DNA sequence according to the invention is accomplished by well known methods that typically depend on the type of vector

used. With regard to these methods, see for example, *Maniatis et al. 1982*, Molecular Cloning, A laboratory Manual, Cold Spring Harbor Laboratory and commercially available methods.

The chimeric inhibitor proteins disclosed herein are preferably produced, recombinantly, in a cell expression system.

5 A wide variety of unicellular host cells are useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, such as CHO, YB/20, NSO, SP2/0, R1. 1, B-W and L-M cells, African Green Monkey kidney cells (e. g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e. g., Sf9), and
10 human cells and plant cells in tissue culture. Preferably, the host cell is a bacterial cell, more preferably an *E. coli* cell.

The present invention is also directed to a pharmaceutical composition comprising the chimeric inhibitor protein as described herein as an active agent, optionally in combination
15 with one or more pharmaceutically acceptable carriers.

Preferably, in addition to at least one chimeric inhibitor protein as described herein, the pharmaceutical composition may contain one or more pharmaceutically acceptable carriers, such as excipients, carriers and/or auxiliaries which facilitates processing of the active compounds into preparation which can be used pharmaceutically.

20 Acceptable carriers, excipients, or stabilizers are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl orbenzyl alcohol; alkyl parabens such as
25 methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating
30 agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN®, PLURONICS® or polyethylene glycol (PEG).

The form of administration of the pharmaceutical composition may be systemic or topical. For example, administration of such a composition may be various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, buccal routes or via an implanted device, and may also be delivered by peristaltic means.

The pharmaceutical composition comprising a chimeric inhibitor protein, as described herein, as an active agent may also be incorporated or impregnated into a bioabsorbable matrix, with the matrix being administered in the form of a suspension of matrix, a gel or a solid support. In addition the matrix may be comprised of a biopolymer.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and [γ] ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT(TM) (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished for example by filtration through sterile filtration membranes.

It is understood that the suitable dosage of a chimeric inhibitor protein of the present invention will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any and the nature of the effect desired.

The appropriate dosage form will depend on the disease, the chimeric inhibitor protein, and the mode of administration; possibilities include tablets, capsules, lozenges, dental pastes, suppositories, inhalants, solutions, ointments and parenteral depots.

Since amino acid modifications of the amino acids of the chimeric inhibitor protein are also encompassed in the present invention, this may be useful for cross-linking the chimeric inhibitor protein to a water-insoluble matrix or the other macromolecular carriers, or to improve the solubility, adsorption, and permeability across the blood brain barrier. Such

modifications are well known in the art and may alternatively eliminate or attenuate any possible undesirable side effect of the protein and the like.

While a preferred pharmaceutical composition of the present invention comprises a chimeric inhibitor protein as an active agent, an alternative pharmaceutical composition may contain a purified and isolated DNA sequence encoding the chimeric inhibitor protein of a protease, as described herein, as an active agent. This pharmaceutical composition may include either the sole purified and isolated DNA sequence, an expression vector comprising said purified and isolated DNA sequence or a host cell previously transfected with an expression vector described herein. In this latter example, host cell will preferably be isolated from the patient to be treated in order to avoid any antigenicity problem. These gene and cell therapy approaches are especially well suited for patients requiring repeated administration of the pharmaceutical composition, since the said purified and isolated DNA sequence, expression vector or host cell previously transfected with an expression vector can be incorporated into the patient's cell which will then produce the protein endogenously.

The present disclosure also provides a method of treating or preventing a proteolysis-associated disorder in a mammal comprising administering to said mammal the pharmaceutical composition as described herein.

The present method of treating or preventing a proteolysis-associated disorder can be useful in case the disorder is a disorder in which hK2 kallikrein activity is detrimental such as a cancer, an autoimmune disorder, an inflammatory disorder such as Benign Prostatic Hypertrophy, or an infectious disorder.

The term "cancer" refers to or describes the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancers, which can be treated, include but are not limited to prostate cancer, breast cancer or a metastatic cancer.

In preferred methods, the mammal is a human patient, and the administered chimeric inhibitor protein is selected from the recombinant serpin examples of Table III, which specifically inhibits the hK2 protease.

Embraced by the scope of the present invention is also the use of the pharmaceutical composition described herein for the preparation of a medicament for the treatment or

prevention of a proteolysis-associated disorder in a mammal in case the disorder is a disorder in which hK2 kallikrein activity is detrimental such as a cancer, an autoimmune disorder, an inflammatory disorder such as Benign Prostatic Hypertrophy, or an infectious disorder.

5 Examples of cancers include but are not limited to prostate cancer, breast cancer or a metastatic cancer.

The chimeric inhibitor proteins of the invention will generally be used in an amount to achieve the intended purpose. For use to treat or prevent a disorder, the chimeric inhibitor proteins or the pharmaceutical compositions thereof, are administered or applied in a therapeutically effective amount. A "therapeutically effective amount" is an amount effective
10 to ameliorate or prevent the symptoms, or prolong the survival of, the subject being treated. Determination of a therapeutically effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure provided herein.

For systemic administration, a therapeutically effective amount or dose can be estimated initially from *in vitro* assays. For example, a dose can be formulated in animal
15 models to achieve a circulating concentration range that includes the IC₅₀ as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

Initial doses can also be estimated from *in vivo* data, e.g. animal models, using techniques that are well known in the art. One ordinarily skill in the art could readily optimise
20 administration to humans based on animal data and will, of course, depend on the subject being treated, on the subject's weight, the severity of the disorder, the manner of administration and the judgement of the prescribing physician.

The present invention also encompasses a method for producing a chimeric inhibitor
25 protein of a protease, said method comprising the steps of

- a) selecting a polynucleotidic sequence encoding a substrate-enzyme interaction site specific for a protease,
- b) introducing said polynucleotidic sequence into a sequence encoding an inhibitor protein of a serine or cysteine protease, so as to obtain a chimeric sequence,
- 30 c) allowing expression of said chimeric sequence in a cell expression system under suitable conditions,
- d) and recovering the chimeric inhibitor protein of a protease.

Selecting a polynucleotidic sequence encoding a substrate active site specific for a protease can be done by the following different techniques such as, for example, displaying substrates for protease selection such as a murine leukemia retrovirus displaying a peptide directly from living cells thus avoiding passage in bacteria (Buchholz et al., 1998) or a similar method using chimeric Sindbis virus libraries which was also employed for the in vivo selection of protease cleavage sites using mammalian cells transfected with the enzyme of interest (*Pacini et al*, 2000 "In vivo selection of protease cleavage sites by using chimeric Sindbis virus libraries" *J. Virol.* 74, 22: 10563-70).

Also envisioned is a yeast system, GASP (genetic assay for site specific proteolysis) which consists in fusing random substrates to an integral membrane protein, allowing the attachment of the substrate to the membrane yeast, where cytoplasmic transcription factors can bind to promoter of a reporter gene (*Kang et al.*, 2001 "An improved strategy for a genetic assay for site-specific proteolysis" *Mol. Cell.* 30; 11(2): 263-6).

Recently have emerged a number of combinatorial chemical libraries for determining protease substrate specificity. These include combinatorial fluorogenic substrate libraries and positional scanning-synthetic combinatorial libraries .

Another method, named immobilized peptide library, allows determination of relative substrate specificity (k_{cat}/K_m) for each member of the library by measuring fluorescence intensity in the solution phase and to identify the scissile bond by Edman sequencing (*Hu et al.* 2002 "Rapid determination of substrate specificity of clostridium histolyticum beta-collagenase using an immobilized peptide library" *J. Biol. Chem.* 8, 277 (10):8366-71).

In case substrate specificity of a protease is determined by phase display technology, a phage-displayed random peptide library with exhaustive diversity is generated and screened with purified protease. This known technique has been adapted to the specific case as described herein in order to construct a phage-displayed random library that included all possible amino acid combination of a defined length of amino acids. Thus large libraries are constructed by displaying random sequences on the extremity of filamentous phages, then amplified and screened toward a protease to assay rapidly its specificity.

According to Examples 1 and 2, Applicants have constructed a pentamer library containing 1.8×10^8 independent transformants which could then be considered complete because, in theory all of the 3.2×10^6 possible random pentamer sequences were represented. The sequences of phages further confirmed the randomness of the pentamer inserts. Then
5 phage displaying the random pentapeptides are fused to a ligand (6x His) and are immobilized on an affinity support, in this case Ni-NTA matrix. Following incubation with the protease, (in the case of examples 1 and 2 the protease was hK2) phages expressing sensitive substrates are released from the solid phase. The released phages are used to infect F-positiv bacteria to be titrated and amplified. These phages are then purified by precipitation, amplified and then
10 immobilized to affinity support to proceed for a next round of selection. This selection of pentapeptides has been repeated 8 times in total in order to obtain high specific polynucleotidic sequence. Phages from the last round are cloned by plating onto Petri dishes and DNA of individual phages is amplified in region encoding a substrate active site to determine the sequences cleaved by the enzyme.

15 Polynucleotidic sequences encoding a substrate active site are then introduced into a sequence encoding an inhibitor of a serine protease, for example into a sequence encoding rACT, so as to obtain a chimeric sequence. Two silent restriction sites *Sac II* and *Mlu I* previously incorporated 18 bp upstream and 18 bp downstream of P1 codon in RSL domain of
20 rACT allowed the subcloning of the selected polynucleotidic sequence encoding a substrate active site.

Recombinant chimeric inhibitors are, for example, produced in TG1 E.coli strains at suitable culturing conditions. Suitable culturing conditions can be comprised between 10-
25 40°C during 10-30 hours depending on the recombinant chimeric inhibitors to be expressed. Surprisingly, Applicants have shown that in the case of examples 1 and 2, a temperature of 16°C during 16h allows the expression and the production of fully intact variants of rACTs.

Finally, recombinant chimeric inhibitors can be recovered either from the culturing
30 medium, when the recombinant chimeric inhibitor is secreted, or extracted from the cell expression system when the recombinant chimeric inhibitor is not secreted, and purified by art-known techniques such as high performance liquid chromatography, gel electrophoresis,

affinity chromatography, ultrafiltration, ion exchange and the like. The actual conditions used to purify a particular recombinant chimeric inhibitor will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, etc. and will be apparent to those skilled in the art.

For affinity chromatography purification, any antibody which specifically binds to the recombinant chimeric inhibitor or to the His tag may be used. Other affinity molecules such as Ni^{2+} -nitrilotriacetic linked to agarose beads and which bind specifically to the His tag are also envisioned in the present invention.

The chimeric inhibitor proteins may then further be assayed for their ability to inhibit the activity of the protease. This can be done by any conventional method such as the Scatchard method (*Scatchard, 1949 Ann NY Acad Sci* 51: 660-669). This method describes a classical method of measuring and analysing binding which has been applied to the binding of proteins and requires relatively pure protein and the ability to distinguish bound protein from unbound.

A second method appropriate for measuring the affinity of chimeric inhibitor proteins for enzymes is to measure the ability of the chimeric inhibitor proteins to slow the action of the enzyme. This method requires, depending on the speed at which the enzyme cleaves substrates and the availability of chromogenic or fluorogenic substrates relatively pure chimeric inhibitor proteins.

Preferably, the chimeric inhibitor proteins of the present invention inhibit the protease activity with a higher affinity than their wild type counterparts.

The chimeric inhibitor proteins disclosed herein are preferably produced, recombinantly, in a cell expression system. This system can be a eukaryotic or a prokaryotic host cell.

A wide variety of unicellular host cells are useful in expressing the chimeric inhibitor proteins of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, such as CHO, YB/20, NSO, SP2/0, R1. 1, B-W and L-M cells, African Green Monkey kidney cells (e. g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e. g., Sf9), and human cells and plant cells in tissue culture. Preferably, the host cell is a bacterial cell selected from the group comprising the genera *Bacillus*, *Escherichia*, *Salmonella*, and

Erwinia. More preferably the bacterial host cell is an *E. coli* cell.

Transformation or transfection of appropriate eukaryotic or prokaryotic host cells with an expression vector comprising a purified and isolated DNA sequence according to the invention is accomplished by well known methods that typically depend on the type of vector used. With regard to these methods, see for example, *Maniatis et al. 1982*, Molecular Cloning, A laboratory Manual, Cold Spring Harbor Laboratory and commercially available methods.

A further object of the present invention is to provide a diagnostic kit for the detection of a protease, *in vivo* or *in vitro*, in a specimen comprising a purified and isolated DNA sequence selected from the group comprising SEQ ID N° 1, 3, 5, 7, 9, 11, 13, a sequence complementary thereof, fragments thereof, and/or variants thereof.

Alternatively, the present invention also envisioned a diagnostic kit for the detection of a protease in a specimen comprising a chimeric inhibitor protein of a protease according to the present invention. Said chimeric inhibitor protein of a protease may, for example, be selected from the group comprising MD820, MD 62, MD 61, MD67 and MD CI.

As used herein, the term "specimen" refers to any suitable sample that may contain a protease, or a sequence encoding for a protease, to which may bind the chimeric inhibitor protein or the purified and isolated DNA sequence encoding for said chimeric inhibitor protein.

The diagnostic kit may include a system enabling the detection of a protease wherein detection of the signal will depend on the amount of protease present. The signal may be detected visually or instrumentally. Possible signals may include production of coloured, fluorescent, or luminescent products, alteration of the characteristics of absorption or emission of radiation by an assay component or product, and precipitation or agglutination of a component product. Said component may be a label, e.g. a radioisotope, a fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, or an agglutinable particle and may be coupled either to the chimeric inhibitor protein or to the purified and isolated DNA sequence present in this diagnostic kit.

Finally, the present disclosure also provides a method of treating or preventing a proteolysis-associated disorder in a mammal comprising administering to said mammal a pharmaceutical composition comprising a recombinant wild type serpin as an active agent.

The aforementioned method of treating or preventing a proteolysis-associated disorder can be useful in case the disorder is a disorder in which hK2 kallikrein activity is detrimental such as a cancer, an autoimmune disorder, an inflammatory disorder such as Benign Prostatic Hypertrophy, or an infectious disorder.

The foregoing description will be more fully understood with reference to the following Examples. Such Examples, are, however, exemplary of methods of practising the present invention and are not intended to limit the scope of the invention.

EXAMPLES

Example 1:

Development of recombinant ACT inhibitors specific to human hK2 using phage display selected substrates.

Material

hK2 and hK3 (PSA) were purified from human semen as previously described (Frenette G, Gervais Y, Tremblay RR, Dube JY. 1998 "Contamination of purified prostate-specific antigen preparations by kallikrein hK2" *J Urol* 159, 1375-8), anti-hK2 and anti-PSA monoclonal antibodies were a gift from Professor RR Tremblay, Laval University, Canada. Human chymotrypsin (Chtr), urokinase plasminogen activator (uPA), human kallikrein hK1, human plasma kallikrein (PK), human neutrophil elastase (HNE) and commercial ACT (human plasma α 1-antichymotrypsin) were purchased from Calbiochem. Z-Phe-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Gly-Gly-Arg-AMC, MeOSuc-Ala-Ala-Pro-Val-AMC were purchased from Calbiochem. CFP-TFRSA-YFP fluorescent substrate was developed as previously described (Mahajan NP et al. 1999 "Novel mutant green fluorescent protein protease substrates reveal the activation of specific caspases during apoptosis" *Chem Biol* 6, 401-9). The cDNA for human α 1-antichymotrypsin (ACT) was a generous gift from Dr. Harvey Rubin (University of Pennsylvania).

Site-directed Mutagenesis

Following the subcloning of ACT cDNA into pQE-9 expression vector (Qiagen, Germany, figure 9) and the introduction of an His₆ tag at the N-terminal of rACT_{WT}, two restriction sites
5 *Sac* II and *Mlu* I, were incorporated 18 bp upstream and 18 bp downstream of P1 codon in
RSL domain respectively. These sites were created by silent mutation using oligonucleotides
5'-GTGATTTTGACCGCGGTGGCAGCAG-3' for *Sac* II and 5'-
GCACAATGGTACGCGTC TCCACTAATG-3' for *Mlu* I site and following the quickchange
mutagenesis protocol supplied by Stratagene.

Construction of the substrate phage display library

Substrate phage libraries were generated using a modified pH0508b phagemid (Lowman *et al.*
1991 "Selecting high-affinity binding proteins by monovalent phage display" *Biochemistry* 12,
10832-8). The construction consists of a His₆ tag at either end of a Gly-Gly-Gly-Ser-repeat-
15 rich region that precedes the carboxyl-terminal domain (codons 249-406) of the M13 gene III.
The random pentamers were generated by PCR extension of the template oligonucleotides
with appropriate restriction sites positioned on both side of the degenerate codons:

5'TGAGCTAGTCTAGATAGGTGGCGGTNNSNNSNNSNNSNNSGGGTCGACGTCGGT
CATAGCAGTCGCTGCA-3' (where N is any nucleotide and S is either G or C) using

5' biotinylated primers corresponding to the flanking regions:

5'TGAGCTAGTCTAGATAGGTG-3' and 5'-TGCAGCGACTGCTATGA-3'.

PCR templates are digested and purified as described previously (Smith G.P, Scott J.K. 1993
"Libraries of peptides and proteins displayed on filamentous phage" *Methods Enzymol.* 217,
228-57), inserted into XbaI/SalI digested pH0508b vector, and electroporated into XL1-Blue

25 (F⁻). The extent of the library was estimated from the transformation efficiency determined
by plating a small portion of the transformed cells onto Luria-Bertani plates containing
ampicillin and tetracycline (100 and 15 µg·mL⁻¹, respectively). The rest of the transformed
cells were used to prepare a phage library by incubating overnight by adding an M13K07
helper phage at a concentration giving a multiplicity of infection of 100 plaque forming units
30 (p.f.u.) per mL. Phages were collected from the supernatant and purified by poly(ethylene
glycol) precipitation. Of these, 200 clones were selected arbitrarily for sequencing to verify the
randomization of the library.

Phage-displayed pentapeptide library screening

This new pentapeptide library was subjected to eight rounds of screening with hK2. One hundred microliters of Ni^{2+} -nitrilotriacetic acid coupled to sepharose beads (Ni^{2+} -nitrilotriacetic acid resin) was washed with 10 mL NaCl/Pi containing $1 \text{ mg}\cdot\text{mL}^{-1}$ BSA.

- 5 Phage particles (10^{11}) were added to the equilibrated Ni^{2+} -nitrilotriacetic acid resin and allowed to bind with gentle agitation for 3 h at 4°C . The resin was subsequently washed ($\text{NaCl}/\text{Pi}/\text{BSA}$ $1 \text{ mg}\cdot\text{mL}^{-1}$, 5 mM imidazole, 0.1% Tween 20) to remove unbound phages and then equilibrated in NaCl/Pi . The substrate phage was exposed to 27 nM (final concentration) of hK2 for 45 min at 37°C . A control selection without protease was also performed. The
- 10 cleaved phages released into the supernatant were amplified using XL1-Blue Escherichia coli and then used for subsequent rounds of selection. After eight rounds of panning, about 15 individual clones were picked from the fifth, sixth and eighth round of selection and plasmid DNA were isolated and sequenced in the region encoding for the substrate.

15 **Construction and Expression of recombinant wild type ACT and its variants.**

Six variants, which correspond to a change in the reactive site loop in positions between P3 and P3' (see Table IV below), were generated by PCR extension of the template oligonucleotides:

- rACT_{8,20}, 5'-TACCGCGGTCAAAATCACCCCTCCGTTCTCGAGCAGTGGA
 20 GACGCGT GA-3';
- rACT_{6,3}, 5'-TACCGCGGTCAAAATCACCAGGAGGTCTATCGATGT
 GGAGACGCGTGA-3';
- rACT_{8,3}, 5'-TACCGCGGTCAAAATCAGGGGGAGATCTGAGTTAGTG
 GAGACGCGTGA-3';
- 25 rACT_{6,7}, 5'-TACCGCGGTCAAAATCAAAGCTTAGAACAACATTAG
 TGGAGACCGCTGA-3';
- rACT_{6,1}, 5'-TACCGCGGTCAAAATCATGACAAGATCTAACTTAGT
 GGAGACGCGTGA-3';
- 30 rACT_{5,18}, 5'-TACCGCGGTCAAAATCACCGAGCGTGTCTCGCCCGTG
 GAGACGCGTGA-3'

(where underlined sequences encode new cleavage sites in the reactive site loop), using primers corresponding to the flanking regions : 5'-TACCGCGGTCAAAATC-3' and 5'-

TCACGCGTGTCCAC-3'. PCR products were digested with *Sac II* and *Mlu I* restriction enzymes and then subcloned into digested rACT_{WT} construct. Recombinant serpins were produced in TG1 *E. coli* strain. Cells were grown at 37°C in 2 x TY media (16g tryptone, 10g yeast extract, 5g NaCl per L) containing 100µg/ml ampicillin to A₆₀₀=0.5. Isopropylthio-β-galactoside (IPTG) was then added to a final concentration of 0.5mM allowing the expression of recombinant serpins for 16h at 16°C. The cells from 100ml of culture were harvested by centrifugation, resuspended in cold PBS and then passed through a french press to recover the total soluble cytoplasmic proteins. Cell debris were removed by centrifugation and Ni²⁺-nitrilotriacetic affinity agarose beads were added to supernatant for 90 min at 4°C to bind recombinant serpins. The resin was subsequently washed with 50mM Tris pH 8.0, 500mM NaCl, 25mM Imidazole and the bound proteins were eluted for 10min with 50mM Tris pH 8.0, 500mM NaCl and 150mM Imidazole. Once purification was completed, rACT were dialysed against 50mM Tris pH 8.0, 500mM NaCl, 0,05 % Triton X-100 for 16h at 4°C. The protein concentration was determined for each purification by Bradford assay and normalized by densitometry of Coomassie Blue-stained SDS-PAGE gels (*Laemmli UK. 1970 "Cleavage of structural proteins during the assembly of the head of bacteriophage T4" Nature 227, 680-5*).

Table IV

Alignment of RSL (Reactive Serpin Loop) of recombinant serpin α1-antichymotrypsin (ACT) and its variants.

| <i>Serpin</i> | <i>Selected^a Substrate Peptide</i> | <i>P6</i> | <i>P5</i> | <i>P4</i> | <i>P3</i> | <i>P2</i> | <i>P1</i> | <i>P'1</i> | <i>P'2</i> | <i>P'3</i> | <i>P'4</i> | <i>P'5</i> | <i>P'6</i> |
|----------------------|---|-----------|-----------|-----------|-----------|-----------|-----------|------------|------------|------------|------------|------------|------------|
| rACT _{WT} | | V | K | I | T | L | <i>L*</i> | S | A | L | V | E | T |
| rACT _{8.20} | LR↓SRA | V | K | I | T | <u>L</u> | <u>R*</u> | S | <u>R</u> | <u>A</u> | V | E | T |
| rACT _{6.2} | RR↓SID | V | K | I | T | <u>R</u> | <u>R*</u> | S | <u>I</u> | <u>D</u> | V | E | T |
| rACT _{8.3} | RGR↓SE | V | K | I | <u>R</u> | <u>G</u> | <u>R*</u> | <u>S</u> | <u>E</u> | L | V | E | T |
| rACT _{6.7} | KLR↓TT | V | K | I | <u>K</u> | <u>L</u> | <u>R*</u> | <u>T</u> | <u>T</u> | L | V | E | T |
| rACT _{6.1} | MTR↓SN | V | K | I | <u>M</u> | <u>T</u> | <u>R*</u> | <u>S</u> | <u>N</u> | <u>A</u> | V | E | T |
| ACT _{5.18} | ER↓VSP | V | K | I | T | <u>E</u> | <u>R*</u> | <u>V</u> | <u>S</u> | <u>P</u> | V | E | T |

^a Substrate peptides selected by kallikrein hK2 using a phage-displayed random pentapeptide library. Plain type residues are common to rACT_{WT}, bold and underlined residues correspond to substrate peptides relocated in RSL of ACT variants. The scissile bond by hK2 in substrate peptides is designated by ↓ and putative cleavage site in serpins is marked by asterisks between the P1-P1' residues.

Inhibition assays and Stoichiometry of inhibition (SI)

The stoichiometry of inhibition (SI) values were determined for the inhibition of rACT_{WT} and its variants with hK2 and different other enzymes. An initial test was made with a molar excess of rACT (100 fold) over hK2, PSA, hK1, chymotrypsin (Chtr), plasma kallikrein (PK), urokinase (uPA) and human neutrophil elastase (HNE) enzymes. The reaction was carried out for 30min at 25°C (90 min at 37°C for PSA) in reaction buffer (50mM Tris pH 7.5, 150mM NaCl, 0,05% Triton X-100, 0,01% BSA) and residual enzyme activity was measured by adding fluorescent substrates (Z-Phe-Arg-AMC for hK1, hK2 and PK, Suc-Ala-Ala-Pro-Phe-AMC for Chtr, , Z-Gly-Gly-Arg-AMC for uPA, MeOSuc-Ala-Ala-Pro-Val-AMC for HNE, and CFP-TFRSA-YFP for PSA). Activity of enzyme in presence of inhibitors was compared to uninhibited reaction. For reactions where an inhibition was observed, SI was determined by incubating different concentrations of recombinant serpins. Using linear regression analysis of fractional activity (velocity of inhibited enzyme reaction / velocity of uninhibited enzyme reaction) *versus* the molar ratio of the inhibitor to enzyme ($[I_0]/[E_0]$), the stoichiometry of inhibition, corresponding to the abscissa intercept, was obtained.

Kinetics

The association rate constants for interactions of hK2, chymotrypsin, PK and HNE with different rACTs were determined under pseudo-first order conditions using the progress curve method (*Morrison JF, Walsh CT. 1988 "The behavior and significance of slow-binding enzyme inhibitors" Adv. Enzymol. Relat. Areas Mol. Biol 61, 201-301*). Under these conditions, a fixed amount of enzyme (2 nM) was mixed with different concentrations of inhibitor (0-800 nM) and an excess of substrate (10μM). Each reaction was made in reaction buffer (50mM Tris pH 7.5, 150mM NaCl, 0,05% Triton X-100, 0,01% BSA) at 25°C for 45min and the rate of product formation was measured using a FLx800 fluorescence 96-well microplate reader (Biotek, USA). In this model, inhibition is considered to be irreversible over the course of reaction and the progress of enzyme activity is expressed by product formation (P), beginning at a rate (v_z) and is inhibited over time (t) at a first-order rate (k_{obs}), rate constant that is dependent only on inhibitor concentration.

$$P = (v_z/k_{obs}) \times [1 - e^{(-k_{obs}t)}]$$

eq 1

For each inhibitor, a k_{obs} was calculated, for four different concentrations of inhibitors, by non linear regression of the data using equation 1. By plotting the k_{obs} versus inhibitor concentration $[I]$, a second-order rate constant, k' , equal to the slope of the curve ($k' = \Delta k_{\text{obs}} / \Delta [I]$), was determined. Due to the competition between inhibitor and the substrate, equation 2 below is used to correct the second order rate constant k' by taking in account the substrate concentration $[S]$ and the K_m of the enzyme for its substrate, giving the k_a .

$$k_a = (1 + [S] / K_m) \times k' \quad \text{eq 2}$$

- 10 The K_m of hK2 for Z-FR-AMC, chymotrypsin for Suc-AAPF-AMC, PK for Z-FR-AMC and HNE for MeOSuc-AAPV-AMC were 67 μ M, 145 μ M, 170 μ M and 130 μ M respectively.

Western blot analysis of complex formation and inhibitor degradation.

- 15 Kallikrein hK2 was incubated 3 hours at 37°C with different recombinant ACTs at a $[I]_0:[E]_0$ ratio of 100:1 in 50mM Tris, 200mM NaCl, 0,05% Triton X-100. Protein samples were heated at 95°C for 5min, separated by SDS-PAGE (12% acrylamid 19:1 T:C ratio) and then electroblotted onto Hybond-ECL (Amersham Pharmacia) nitrocellulose. The free-hK2 and hK2-ACT complexes were detected using a mouse anti-hK2 monoclonal antibody and an
20 alkaline phosphatase-conjugated goat anti-mouse secondary antibody. Western blot was visualized using the ECL detection kit (Amersham Pharmacia Biotech). hK2 was also incubated with ACT_{8.3} or ACT_{6.7} 30min at 25°C (kinetic conditions) at a $[I]_0:[E]_0$ ratio of 10:1 in 50mM Tris, 200mM NaCl, 0,05% Triton X-100. Proteins were detected by western blot, using an anti-His₆ monoclonal antibody followed by detection with the secondary antibody
25 and protocol described above.

Production of soluble recombinant wild type and variant ACTs

- Wild type serpin $\alpha 1$ -antichymotrypsin was used to develop specific inhibitors of the kallikrein hK2. Residues P3-P3' located in RSL structure of rACT_{WT} were replaced by substrate
30 pentapeptides, previously selected by phage display technology as described above. Six variants of rACT shown in table I, have been designed and constructed. The scissile bond in substrate peptides was aligned according to Leu-358-Ser-359 into RSL of the serpin. rACT_{WT}

and its variants were expressed in *E.coli* TG1 as fusion proteins containing an His tag in N-terminal position. Each of them was produced at low temperature allowing protein accumulation mainly in active soluble form. Purified under native conditions, the level of production varied between 1.0 to 2.5 mg/L. The purity of purified serpins, such as for example Variant 6.1 (lane 1) and wild type ACT (lane 2), as estimated by SDS-PAGE analysis is more than 98% (figure 1).

rACT variants are mainly specific to kallikrein hK2

A panel of enzymes including human neutrophil elastase, chymotrypsin-like (Chtr, PSA or hK3) and trypsin-like (hK2, hK1, PK, uPA) proteinases have been screened to determine inhibitory specificity of rACT variants (Table V).

Table V

Inhibitory profile of rACT_{WT} and its variants.

| | ACT _{8.20} (LR↓SRA) ^a | ACT _{6.2} (RR↓SID) ^a | ACT _{8.3} (RGR↓SE) ^a | ACT _{6.7} (KLR↓TT) _n | ACT _{6.1} (MTR↓SN) _n | ACT _{5.18} (ER↓VSP) ^a | ACT _{WT} (LL↓SA) _n |
|--------------|--|---|---|---|---|--|---|
| | MD 820 | MD 62 | MD 83 | MD 67 | MD 61 | MD518 | |
| Protease | Inhibition % ^b | | | | | | |
| hK2 | 95 | 100 | 100 | 100 | 100 | 73 | 0 |
| Chtr | 66 | 0 | 0 | 0 | 0 | 0 | 100 |
| PK | 54 | 100 | 0 | 36 | 100 | 0 | 0 |
| HNE | 30 | 0 | 0 | 0 | 60 | 0 | 15 |
| PSA (hK3) | 45 | 0 | 0 | 0 | 0 | 0 | 80 |
| hK1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Urokinase | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

^a Amino acid sequence cleaved in RSL (Reactive Serpin Loop) of recombinant ACTs corresponding to selected substrate peptide by hK2.

^b Protease and serpins were incubated for 30 min at 25°C (90 min at 37° for PSA) at a [I]₀/[E]₀ ratio of 100:1. Percent inhibition correspond to 100 x (1- (velocity in presence of inhibitor / velocity of uninhibited control)).

Incubating with an excess of inhibitors ([I]₀/[E]₀ of 100:1) for 30 minutes, hK2 is completely inhibited by rACT_{6.2}, rACT_{8.3}, rACT_{6.7} and rACT_{6.1}, whereas rACT_{8.20} and rACT_{5.18} inhibited

95 % and 73 % of enzyme activity, respectively. Under this condition, wild type rACT showed no inhibition activity toward hK2. Among these variants, two (rACT_{8.3} and rACT_{5.18}) are specific to hK2, inhibiting no other tested enzyme. Two other variants, rACT_{6.7} and rACT_{6.2}, inhibited as well PK at 36 % and 100% respectively. As wild-type ACT, variant rACT_{8.20} inhibited the two chymotrypsin-like proteases Chtr and PSA but additionally also PK and HNE. None of the recombinant serpins showed inhibitory activity against the kallikrein hK1 and uPA.

Stoichiometries of inhibitory of variant ACTs for hK2 are improved drastically in comparison to wild type ACT

The determination of the stoichiometry of inhibitory was accomplished under physiological conditions of pH and ionic strength for all enzymes to ensure the most valuable comparison. Recombinant wild type ACT gave a SI value of 2 (table VI) with chymotrypsin which is identical to the value obtained with commercial ACT under similar conditions (data not shown).

Table VI

Comparison of stoichiometry of inhibition values and second-order rate constants (k_a) for the reaction of rACT_{WT} and its variants with hK2 and others proteinases.

| | ACT _{8.20} (LR↓SRA) ^c MD820 | | ACT _{6.2} (RR↓SID) MD62 | | ACT _{8.3} (RGR↓SE) MD83 | | ACT _{6.7} (KLR↓TT) MD67 | | ACT _{6.1} (MTR↓SN) MD61 | | ACT _{5.18} (ER↓VSP) MD518 | | ACT _{WT} (LL↓SA) c | |
|-----------------|---|-----------------------------|--|---------------------------|--|---------------------------|--|---------------------------|--|---------------------------|--|---------------------------|-----------------------------------|---------------------------|
| | c | | c | | c | | c | | c | | c | | c | |
| | SI | $(k_a)^b$ $M^{-1}s^{-1}$ | SI | k_a^b $M^{-1}s^{-1}$ | SI | k_a^b $M^{-1}s^{-1}$ | SI | k_a^b $M^{-1}s^{-1}$ | SI | k_a^b $M^{-1}s^{-1}$ | SI | k_a^b $M^{-1}s^{-1}$ | SI | k_a^b $M^{-1}s^{-1}$ |
| Protease | | | | | | | | | | | | | | |
| hK2 | 105 | 1779 | 25 | 6261 | 34 | 2439 | 9 | 8991 | 19 | 3442 | 139 | 595 | - | - |
| Chtr | 134 | 905 | - | - | - | - | - | - | - | - | - | - | 2 | 61295 |
| PK | 150 | 424 | 18 | 6217 | - | - | 277 | 201 | 16 | 8024 | - | - | - | - |
| HNE | 334 | 158 | - | - | - | - | - | - | 159 | 1192 | - | - | - | - |

^a SI values reported were determined using linear regression analysis to extrapolate the I/E ratio (see figure 1).

^b Second order rate constants for serpin-proteinase reactions were measured under pseudo-first- or second order conditions as described in "Experimental Procedure".

^c Amino acid sequence of P3-P3' residues in RSL (Reactive Serpin Loop) of recombinant ACT corresponding to selected substrate peptide by hK2

-, No detectable inhibitory activity.

In order to determine the SI values of all the recombinant variants, Applicants have incubated hK2 (5nM) with different concentrations (6.25-500nM) of rACT_{8.20} (x), rACT_{6.2} (□), rACT_{8.3} (Δ), rACT_{6.7} (◇), rACT_{6.1} (⋈), rACT_{5.18} (○), rACT_{WT} (+), at 25°C for 30 min in reaction buffer. Residual activities (velocity) for hK2, were assayed by adding the fluorescent substrate (10μM) Z-FR-AMC. Fractional velocity corresponds to the ratio of the velocity of inhibited enzyme (v_i) to the velocity of the uninhibited control (v_o). The SI was determined using linear regression analysis to extrapolate the I/E ratio (i.e. the x intercept).

As shown in Figure 2 all newly constructed variants of ACT showed lower SI values with hK2 than wild type ACT. From these variants rACT_{6.7}, rACT_{6.1} and rACT_{6.2} had the lowest stoichiometry of inhibition values for hK2 (9, 19 and 25 respectively). Whereas rACT_{6.2} and rACT_{6.1} had also the lowest SI values (18 and 16) for PK, the SI for rACT_{6.7} was much higher (277). The two recombinant ACTs specific for hK2, rACT_{8.3} and rACT_{5.18} had a higher SI ratio of 34 and 139, respectively. The SI value of rACT_{8.20} inhibitor was superior to 100 for all tested proteases including hK2.

Variant ACTs form stable complexes with hK2 without degradation of inhibitors

hK2 was incubated 3h at 37°C with rACT_{8.20}, rACT_{6.2}, rACT_{8.3}, rACT_{6.7}, rACT_{6.1}, rACT_{5.18} and wild type rACT, at a I:E ratio of 100:1. Western Blot analysis of the reaction products of rACTs with hK2 (rACT_{8.20} (lane 1), rACT_{6.2} (lane 2), rACT_{8.3} (lane 3), rACT_{6.7} (lane 4), rACT_{6.1} (lane 5), rACT_{5.18} (lane 6) and wild type rACT (lane 7)), has been done under reducing conditions using a mouse anti-hK2 antibody to determine the fate of inhibitors after the interaction with the enzyme. Figure 3A shows that when hK2 is incubated with ACT variants, free hK2 (E) disappeared completely to form a covalent complex (EI). This covalent complex demonstrated a high stability as it did not break down over a 16h incubation period (data not shown). Wild type ACT inhibited more slowly hK2, which was mainly uncomplexed after 3 hours of incubation. Elevated SI values measured with hK2 were not due to non-complex forming degradation of ACT variant inhibitors.

Further on ACT_{8.3} (lane 1) or ACT_{6.7} (lane 3) were incubated with hK2 (lane 2 and 4 respectively on the western blot) under kinetic conditions (30min at 25°C) at a I:E ratio of 10:1. The complex formation was analysed by western blot under reducing conditions using a mouse monoclonal anti-his tag (figure 3B). All inhibitor proteins were either complexed with

hK2 or present as uncleaved form, indicating that the possible substrate pathway for the serpin-enzyme interaction is marginal.

Variant ACTs showed highest association constants with hK2

5 The rate of inhibitory reaction with variant ACTs was determined for each protease showing reactivity with these inhibitors. To that end, interaction of hK2 and recombinant serpins was measured under pseudo-first order conditions using progress curve method. hK2 (2nM) and substrate Z-FR-AMC (10 μ M) were added to varying amounts (20n-800nM) of inhibitors rACT_{8.20}(\diamond), rACT_{5.18}(+) (Figure 4A) and inhibitors rACT_{6.2}(\circ), rACT_{8.3}(\square) rACT_{6.7}(Δ),
10 rACT_{6.1}(x) (Figure 4B). Representative progress curves were subjected to non linear regression analysis using eq 1 and the rate (k_{obs}) was plotted against the serpin concentrations. After determination of k_{obs} , association constants (ka) were calculated using K_m of the proteases for their corresponding substrates (table VI). The ka value of wild type ACT with chymotrypsin was identical as to published data (Cooley *et al.* 2001 "The serpin MNEI
15 inhibits elastase-like and chymotrypsin-like serine proteases through efficient reactions at two active sites" *Biochemistry* 40, 15762-70). The recombinant rACT_{6.7} showed a highest ka (8991 M⁻¹s⁻¹) with hK2 whereas that obtained with PK was 45 fold inferior. In contrast, recombinant rACT_{6.2} gave equivalent ka with hK2 and PK demonstrating a lack of discrimination between the two proteases. ka values of hK2 specific recombinant inhibitors
20 rACT_{8.3} and rACT_{5.18} were lower, 2439 and 595 M⁻¹s⁻¹ respectively, whereas non specific ACT_{8.20} exhibited a ka of 1779 M⁻¹s⁻¹, for hK2, superior compared to Chtr, PK and HNE. One of the recombinant serpins, rACT_{6.1}, was reacting at higher velocity with PK than with hK2.

Example 2:

25 **Development of recombinant ACT inhibitors specific to human hK2 and hK3 proteases.**

Residues P3-P3' located in RSL structure of rACT_{WT} were replaced by substrate pentapeptide coding for the RSL of Protein C Inhibitor (PCI) (Table VII) as described in example 1.

Table VII

Alignment of RSL (Reactive Serpin Loop) of recombinant serpins ACT, PCI and ACT_{PCI}.

| <i>Serpin</i> | | <i>RSL sequences</i> | | | | | | | | | | | |
|--------------------------------------|----------------------|----------------------|-----------|-----------|-----------|-----------------|-------------------|-----------------|-----------------|-----------------|------------|------------|------------|
| | | <i>P6</i> | <i>P5</i> | <i>P4</i> | <i>P3</i> | <i>P2</i> | <i>P1</i> | <i>P'1</i> | <i>P'2</i> | <i>P'3</i> | <i>P'4</i> | <i>P'5</i> | <i>P'6</i> |
| rACT_{WT} | Amino acid sequence | V | K | I | T | L | L | S | A | L | V | E | T |
| | DNA sequence (codon) | GTC | AAA | ATC | ACC | CTC | CTT | TCT | GCA | TTA | GTG | GAG | GTC |
| rPCI_{WT} | Amino acid sequence | T | I | F | T | F | R | S | A | R | L | N | S |
| rACT_{PCI} (MD CI) | Amino acid sequence | V | K | I | T | <u>F</u> | <u>R</u> | <u>S</u> | <u>A</u> | <u>L</u> | V | E | T |
| | DNA (codon) | GTC | AAA | ATC | ACC | TTT | <u>AGA</u> | TCT | GCA | TTA | GTG | GAG | GTC |

Plain type residues are common to rACT_{WT}, bold and underlined residues correspond to substrate peptides relocated in RSL of ACT variants. The scissile bond in substrate peptides is designated by ↓ and putative cleavage site in serpins is marked by asterisks between the P1-P1' residues.

Briefly, to produce the recombinant protein ACT_{PCI} (MDCI), TG1 cells were transformed with the corresponding constructions followed by growth in appropriate culture media. Cells were then induced to an optimal density to express recombinant inhibitors for 16h at 16°C. Recombinant inhibitor ACT_{PCI} was extracted from cytoplasm bacteria and separated by affinity chromatography using Ni-NTA column as described for the previous example.

Analysis of recombinant ACT expression by SDS-PAGE.

The purity of the different inhibitors developed in example 1 and 2 was tested by SDS-PAGE analysis under reducing conditions as shown in Figure 5.

Evaluation of the inhibitors.

These inhibitors were further analysed to assess their specificity and affinity to inhibit the human kallikreins hK2 and hK3 (Figures 6) and plasma kallikrein, trypsin, urokinase, elastase, thrombin, hK14 and human kallikrein 8 (Table VIII). These two enzymes possess different

enzymatic specificities (hK2: trypsin-like, hK3: chymotrypsin-like) but are naturally inhibited by ACT. While ACT is considered to be the natural hK3 inhibitor in blood circulation, its inhibition of hK2 is weaker.

- 5 Analysis of the inhibitory reaction between rACTs and the human kallikreins were analysed by Western Blot as shown in Figures 6. For each variants of ACT, 1 μ g of inhibitor was incubated with 100 ng of either hK2 or hK3 during 1 hour at 37°C under physiological conditions.
- 10 The results of the detection using the monoclonal antibody anti hK2 9D5 are shown in Figure 6 A.
- Line 1 : hK2 only, 2 : commercial ACT + hK2, 3 : wild type ACT + hK2, 4 : MD820 + hK2, 5 : MDCI + hK2, 6 : MD62 + hK2, 7 : MD61 + hK2,
- Figure 6 B shows the detection of hK3-ACT complex using antibody anti-His (tag present on recombinant ACT proteins).
- 15 Line 1: PSA, 2: PSA + ACT, 3: wild type ACT + PSA, 4 : MD820 + PSA, 5 : MDCI + PSA, 6 : MD62 + PSA, 7 : MD61 + PSA.

The amino acid changes within the reactive loop using substrate sequences selected for hK2 specificity transformed ACT into an inhibitor highly specific for hK2 (MD820, MD61, MD62) without inhibiting hK3. These results confirm those previously shown in Table IV.

20 Only MDCI, based on the reactive loop of the inhibitor of the Protein C (PCI) is able to inhibit both kallikreins tested (hK2 and hK3).

MD61 and MD62 are inhibitors with very high affinity for hK2 inhibiting all hK2 protein in less than 3 minutes (under the same conditions) compared to wild type or commercial α 1-antichymotrypsin, which requires more than 12 hours of incubation to inhibit the same amount of hK2 (data not shown).

25

Table VIII

Inhibitory profile of MD_{CI}.

| Protease | Inhibition % ^b | SI | $k_a \text{ } M^{-1} s^{-1}$ |
|--------------------|---------------------------|------|------------------------------|
| Chymotrypsin | 98 | 1 | 86216 |
| Plasma Kallikrein | 100 | 4.6 | 25900 |
| Trypsin | 100 | 1 | 1126025 |
| Urokinase | 0 | - | - |
| Elastase | 0 | - | - |
| Thrombin | 0 | - | - |
| hK14 | 100 | 3.2 | 287000 |
| Human Kallikrein 8 | ~25 | ~180 | |

Example 3:**Inhibition of tumor growth by MD inhibitors****3.1 Inhibition of tumor growth by MD62 and MD 67 inhibitors**

The androgen-independent human prostate adenocarcinoma cell line DU-145 was obtained from American Type Culture Collection. Retroviral technology was used to obtain DU145 cell transfected with hK2 gene (DU145/hK2).

Exponentially growing DU145/hK2 cells were collected and resuspended at a concentration of 7.5×10^7 cells/ml in DMEM (Invitrogen) containing 1 or 10 μ g of inhibitors. This cell suspension was mixed with matrigel (BD Biosciences) at a 1:2 ratio and injected subcutaneously (3×10^6 cells/40 μ l) into the right and left flank of 8 week old male athymic Swiss nude mice (two mice/group). Each mouse was inoculated at two sites.

At days 6, 12 and 18 following tumor inoculation, 50 or 10 μ g of MD62, MD67 or 100 μ g or 10 μ g of ACT-WT were injected subcutaneously. At days 24, 30, 33, 36, 39 and 41 following tumor inoculation, 25 or 5 μ g of inhibitors (MD62 and MD67) or 50 μ g or 5 μ g of ACT-WT were injected subcutaneously.

5

Figure 10A shows the Inhibition of tumor growth by MD 62. Prostate cancer cells DU-145 (3×10^6 cells), transfected with human kallikrein 2, were implanted in nude mice and then treated with MD 62 (5 or 25 μ g/injection).

10 **Figure 10B** shows the inhibition of tumor growth by MD 67. Prostate cancer cells DU-145 (3×10^6 cells), transfected with human kallikrein 2, were implanted in nude mice and then treated with MD 67 (5 or 25 μ g/injection).

3.2 Inhibition of tumor growth by MDCI inhibitor.

15 The androgen-independent human prostate adenocarcinoma cell line DU-145 was obtained from American Type Culture Collection. Retroviral technology was used to obtain DU145 cell transfected with hK2 gene (DU145/hK2).

Exponentially growing DU145/hK2 cells were collected and resuspended at a concentration of
20 7.5×10^7 cells/ml in DMEM (Invitrogen) containing 1 or 10 μ g of inhibitors. This cell suspension was mixed with matrigel (BD Biosciences) at a 1:2 ratio and injected subcutaneously (3×10^6 cells/40 μ l) into the right and left flank of 8 week old male athymic Swiss nude mice (two mice/group). Each mouse was inoculated at two sites.

At days 6, 12 and 18 following tumor inoculation, 100 μ g or 10 μ g of MDCI or ACT-WT were
25 injected subcutaneously. At days 24, 30, 33, 36, 39 and 41 following tumor inoculation, 50 μ g or 5 μ g of MDCI or ACT-WT were injected subcutaneously.

Figure 11 shows the inhibition of tumor growth by MD CI. Prostate cancer cells DU-145 (3×10^6 cells), transfected with human kallikrein 2, were implanted in nude mice and then treated
30 with MD CI (5 or 50 μ g/injection).